IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	:	
KENSIL et al.	:	
Serial No. 07/200,754	:	Art Unit: 183
Filed: May 31, 1988	:	Examiner: N. Carson
For: SAPONIN ADJUVANT	:	Atty. Dkt.: 0614.0690004

DECLARATION OF DR. CHARLOTTE KENSIL UNDER 37 C.F.R. § 1.132

Honorable Commissioner of
Patents and Trademarks
Washington, DC 20231

Sir:

Charlotte Kensil declares that:

1. I am one of the inventors of the invention disclosed and claimed in United States Application Serial No. 07/200,754.

2. I am the Section Head, Natural Products Chemistry, at Cambridge BioScience Corporation in Worcester, Massachusetts. My c.v. including a list of publications, is attached hereto as Appendix I.

3. I have read and understood the Office Action dated May 22, 1989, and the cited references.

4. Our invention that is claimed in the patent application is to purified saponin adjuvants derived from Quillaja saponaria extract. It is important to note, that with the newly submitted claims, that we are not claiming purified saponins as a class but only those which

SAIDMAN, STERNE,
SSLER & GOLDSTEIN
ATTORNEYS AT LAW
:25 CONNECTICUT AVENUE
ASHINGTON, D. C. 20036
(202) 466-0800

could be classified as adjuvants. Although compounds with the generic properties of saponins (i.e., steroid or triterpene glycosides causing foaminess in aqueous solution) can be isolated from at least 400 species of plants, only a small proportion of these saponins have adjuvant activity. Among those saponins for which there is no report in the literature of adjuvant activity are those mentioned by the Examiner: ginseng saponins by Toyo Jozo, Zhou, and Nagasawa; caullophylogen saponin by Combier; and soybean saponins by Kishimoto Sangyo. Our claimed saponin adjuvants are from Quillaja saponaria are distinguishable from the multiplicity of other plant saponins with different structure, biological properties, and chromatographic behavior. As stated by A.J. George (George, A.J., Food Cosmet. Toxicol. 3:85-91 (1965)):

> Saponins are widely distributed throughout the plant kingdom and have been identified in at least 400 species belonging to 60 different plant families. Common plants which contain saponins include spinach, beetroot, sugar-beet, beech, asparagus, crocus, horsechestnut, alfalfa, and soya.

In the same review, he later states:

> It has been a popular misconception in the past that only one saponin existed. Several papers have been published on the toxicity of "saponin" or "commercial saponin," with no mention of the source. In view of the great variation in the properties and biological effects of saponins from different sources, such papers contribute little toward an understanding of the toxicology of the saponins. "Saponin" can still be seen in some chemical price lists with no indication of its plant of origin.

AIDMAN, STERNE,
SLER & GOLDSTEIN
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25 CONNECTICUT AVENUE
SHINGTON, D. C. 20036
(202) 466-0800

This review was published in 1965. However, even today, Sigma Chemicals offer "saponin" with no description of the source. Thus, saponins and saponin adjuvants from other sources are unlikely to have the same structure and biological properties as the saponin adjuvants from Quillaja saponaria. For example, as discussed in James B. Hudson, Antiviral Compounds from Plants (1990) pages 140-141, copy attached, crude saponin extracts of ginseng, Panax ginseng C.A. Meyer, were found to be immunosuppressive in mice (Yeung et al. (1982) Am. J. Chinese Med. X, 1.

5. The Examiner rejected certain claims as being unpatentable over Higuchi et al. (Phytochemistry 26:229-235) or Scott et al. (Int. Archs. Allergy Appl. Immun. 77:409-412) in combination with Combier et al., Toyo Jozo, Kishimoto Sangyo, Nagasawa et al., or Zhou et al. The Examiner states that "Each of Higuchi et al. and Scott et al. teach the mixture of saponin adjuvants in the bark extract of Quillaja species. That said extract contains a mixture of saponins is well known." We disagree with the Examiner's interpretation of Higuchi and Scott.

Higuchi teaches a mixture of saponins but not of saponin adjuvants in the bark extract of Quillaja. Higuchi states specifically:

> The existence of a saponin mixture (designated as quillajasaponin), which was reported to have a strong adjuvant activity (2,3) and a plasma cholesterol lowering effect (4), was recognized but as for the constituents of the crude saponin, little was known except for quillaic acid (5,6) and its monoglucuronide

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> (7), which were obtained upon acid hydrolysis of the saponin.

There is no reference within this paper to the possibility that multiple saponins would have adjuvant activity, but only that the mixture had adjuvant activity, which could be interpreted in several ways: (1) that only the mixture was adjuvant-active but not necessarily the individual components, or (2) that one component of the mixture was adjuvant-active and was contaminated with the other components, or (3) that multiple components of the mixture and possibly all were adjuvant-active.

Scott states "The adjuvant saponin is a mixture of triterpene glycosides extracted from the tree of Quillaja saponaria (5) and is routinely incorporated in commercial foot-and-mouth disease vaccines." It is unclear from Scott's statement whether he considers that all triterpene glycosides in the extract have adjuvant activity or whether he considers that the adjuvant is one triterpene glycoside which is contaminated with other triterpene glycosides. Hence, it is essential to consider Scott's reference (5) from which he derives his conclusion. This reference is Dalsgaard, K. (1978), Acta vet scand. 69: suppl. pp.1-40. In this reference, Dalsgaard describes the bark extract which serves as his starting material for further purification of the adjuvant-active components:

> Dialyzed quillaja extract was chosen as the starting material for all subsequent purification steps (111). From analytic TLC, it could be seen that at least 5 different substances were present in the dialyzed extract.

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Hence, he does not teach the presence of multiple components, as quoted by Scott. However, his finding was that the adjuvant activity was associated with only one of these bands which he designated as "Quil-A" after carrying out the purification described in this paper. This purification was more completely described in a preceding paper (Dalsgaard, K., Archiv fur die gesamte Virusforschung 44:243-254 (1974)). In this study, Dalsgaard made an aqueous extract of Quillaja bark, subjected it to extended dialysis, and then tested the adjuvant activity of the dialyzed material in comparison to the original aqueous extract. He concluded that:

> Furthermore, it was shown that the crude extract can be dialyzed without loss of activity. Consequently, we prepared a large pool of dialyzed extract and kept it in the freezer at -20°C. This pool served as the starting material for the subsequent purification steps and as reference adjuvant in the immunological experiments presented in this paper. This pool of dialyzed extract is denoted DQ.

Dalsgaard then went on to process the dialyzed extract (DQ) by ion exchange chromatography, gel filtration chromatography, and then again by ion exchange chromatography to obtain a pool designated as fraction F. Of this fraction, he states:

> It will be seen from Table 1 that only peak F had adjuvant activity in guinea pigs. Furthermore, peak F had an activity equivalent to that of the DQ reference, i.e., an approximately fourfold increase in both antibody titre and resistance to challenge as compared to the antigen alone. These results indicated that all the adjuvant activity was present in peak F.

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Hence, Dalsgaard considered that all or almost all of the adjuvant activity which could be extracted into water from Quillaja bark could be isolated in his fraction F which he designated as Quil-A later in the paper. He states:

> Our immunological experiments have not included the investigation of all the substances present in the crude extract of quillaja bark, and thus it cannot be claimed that Quil A is the only substance with adjuvant activity. However, it is considered that Quil A is of special interest because it has the same adjuvant activity as DQ, and because it can be isolated in virtually pure state by relatively simple techniques.

Although Dalsgaard speculates about the possibility of other minor adjuvant fractions, it is clear that he considers Quil-A to be the predominant adjuvant. Hence, no data or statement in Dalsgaard's papers teaches that there are multiple saponin adjuvants. Hence, it is unlikely that Scott was implying that each of multiple triterpene glycosides were adjuvants because this statement is clearly not in the Dalsgaard reference which Scott cited.

6. The Examiner states "It would have been obvious to one of ordinary skill in the art to substantially purify the conventional saponin adjuvant mixture of Scott et al. or Higuchi et al., known to enhance the immune response to antigen, since purification of various saponins is well known and advantageous, as disclosed by each of the secondary references." Again, we disagree with the Examiner's conclusion.

AIDMAN, STERNE,
SLER & GOLDSTEIN
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25 CONNECTICUT AVENUE
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Dalsgaard teaches us the purification of a fraction (designated Quil-A) which he reported as containing almost all of the adjuvant activity of Quillaja bark extract as described above. In addition, he teaches us that Quil-A is a single component (Dalsgaard, K., Archiv fur die gesamte Virusforschung 44:243-254 (1974)):

> However, it is considered that Quil-A is of special interest because it has the same adjuvant activity as DQ, and because it can be isolated in virtually pure state by relatively simple techniques.

The Dalsgaard reference has been widely quoted in the literature. In no paper prior to our patent application has the purity as described by Dalsgaard been questioned. In papers which have reported on toxicity due to Quil-A, the conclusion has been that toxic side effects are due to the single component reported by Dalsgaard rather than one component of a mixture. Our embarking on the saponin purification project meant that we were completely disregarding the teachings of Dalsgaard in reference to the purity of Quil-A and the techniques used to assess purification. In fact, by application of analytical techniques for which we developed the methods, we were able to demonstrate within the patent application (Figure 3) that commercially available Quil-A (reported by the manufacturer to have been prepared by the method of Dalsgaard and to be pure) is not in fact a pure product. Attached as declaration Figure 1 is a recent analytical HPLC utilizing a solvent system which gives a higher resolution to further illustrate the heterogeneity of products within

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25 CONNECTICUT AVENUE
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Quil-A. Three different lots of Quil-A were tested and shown to have a similar level of heterogeneity.

In addition, we have disregarded the teachings of Dalsgaard during the course of our purification. Dalsgaard monitored all peaks at 280 nm. His reason as stated in Acta vet scand. 69: supp. pp. 1-40 (1978) are the following:

> By u.v. scanning on a Guildford spectrophotometer it was estimated that Quil-A has an absorption maximum at 280 nm.

Had we utilized the detection method of Dalsgaard and monitored at 280 nm rather than using refractive index or absorbance at 214 nm, we would have not detected QA-7, QA-17, QA-18, or QA-21. Figure 3 in our patent application shows the UV scans of the purified products, which clearly show the absence of a significant 280 nm absorbance maxima. Only the fractions in and near the solvent peak on the HPLC had UV absorbance at 280 nm (data not shown).

Dalsgaard also rejected the use of silica chromatography as a method of purification of Quillaja saponins in the above reference:

> Since the TLC method (Chapter 3) gave high resolution it was now attempted to adjust this technique to a preparative scale. TLC plates with a layer thickness of 2.5 mm silica gel were run with the analytical solvent system. By scraping and elution of the silica gel, it was possible to isolate chromatographically pure substances. These substances were tested for adjuvant activity in guinea pigs, but none were found effective. It was concluded that either the solvent system was harmful to the immunologically active substance or the recovery from the silica gel was impossible.

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25 CONNECTICUT AVENUE
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(202) 466-0800

Had we followed Dalsgaard's teachings, we hence would have never considered silica chromatography and the use of the organic solvents required to elute saponins from silica. The use of organic solvents was critical to our success as these solvents allowed the dispersal of mixed micelles of saponin into saponin monomers which permitted the separation of individual saponins by adsorption (silica) chromatography and by reverse phase chromatography. Application of adsorption and reverse phase chromatography techniques (as utilized for other saponins described in the secondary references) would clearly not be obvious to a worker familiar with the immunological literature on Quillaja saponins; Dalsgaard is referenced in the overwhelming majority of papers published on adjuvant activity of saponins since the publication of his papers in 1974 and 1978.

The Examiner states that it would have been obvious to apply the techniques in the secondary references. However, purification of Quillaja saponins required extensive effort and skill for the development of effective analytical and preparative chromatography due to the unusually high glycoside content in comparison with other saponins from other sources. The difficulty in purification of Quillaja saponins is illustrated by the lack of success by other groups in purifying individual saponins. Higuchi (Phytochemistry 26:229-235 (1987)) described a purification of Quillaja saponin in which their purest material still yielded 7 spots on silica gel TLC despite two fractionation columns in alcoholic mixtures using conditions in which saponins are dispersed into monomers. The only products which were purified in Higuchi's paper were alkaline

AIDMAN, STERNE,
SLER & GOLDSTEIN
ATTORNEYS AT LAW
5 CONNECTICUT AVENUE
SHINGTON, D. C. 20036
(202) 466-0800

hydrolysis products; our own research as reported in the patent application demonstrates that the major alkaline hydrolysis products have at best a very poor adjuvant activity (less than aluminum hydroxide). In addition, Higuchi does not test or speculate on the use of any of his partially purified mixtures or alkaline-hydrolysis generated byproducts as adjuvants.

7. One of the most important aspects of the invention is that adjuvant fractions are obtained which are substantially pure, boost the humoral immune response, and are safe at adjuvant-effective doses as well as substantially higher doses. For example, with the commercial adjuvant Quil-A, there are reports of toxic effects near the adjuvant effective dose level. Flebbe and Braley-Mullen (Cellular Immunology 99:119-127 (1986)) describe their experience with Quil-A in routine immunization.

> Each animal received 25 ug of Quil-A which was mixed with the antigen prior to injection. Doses ranging from 10 to 100 per mouse were tested in preliminary experiments. Amounts greater than 25 ug per mouse were too toxic for routine use and 100 ug was less effective as an adjuvant.

Sundquist et al. (Vaccine 6:49-53 (1988)) report from their experience in using ISCOMS (micelles prepared from protein and Quil-A) for subcutaneous or intradermal immunization in mice:

> Above this concentration local reactions start to be noticeable; transferred to mice, it would mean at a dose containing about 10 ug Quil-A.

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SLER & GOLDSTEIN
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(202) 466-0800

Severe toxic reactions to Quil-A have also been observed in other species. Allison et al. (J. of Immunol. Methods 95:157-168 (1986)), in a review of adjuvants, report in regard to Quil-A that:

> However, virus vaccines containing small amounts of heterologous serum administered with saponin to cattle have produced severe immediate reactions upon challenge . . . since saponins produce tissue damage at injection sites, do not stimulate the formation of antibodies of isotypes giving optimal protection and elicit reaginic antibodies, their suitability for human use can be questioned.

The Examiner states that "it would have been obvious to the worker in the art to obtain the expected pure saponin having fewer impurities and being less toxic." Our own experience refutes this point. We did not assume that any individual saponin adjuvant would necessarily be detoxified as a result of purification. We recognized that the toxicity associated with a particular saponin and not with another might be a function of subtle structural differences between those saponins. Nor did we assume that adjuvant activity and toxicity are correlated. In the patent application, we identified one toxic saponin QA-19A and recognized the possibility that more might exist. The carbohydrate analysis of this saponin (Table 3, in the patent application) indicates a similar although not identical glycoside structure to the other saponins. Since this time, we have carried out additional toxicity studies to identify other toxic saponins. The data presented below in declaration Table 1 indicate that purified QA-7 is significantly less toxic than the commercial product Quil-A whereas purified QA-18 is significantly more toxic than Quil-A

SAIDMAN, STERNE,
SSLER & GOLDSTEIN
ATTORNEYS AT LAW
125 CONNECTICUT AVENUE
ASHINGTON, D.C. 20036
(202) 466-0800

(although both are apparently nontoxic in an adjuvant-effective dose range). However, both QA-7 and QA-18 appear to have similar effects as adjuvants on boosting humoral immune response to an antigen in mice. Hence, the simplistic prediction that all saponins which are adjuvants are also nontoxic clearly does not apply; an effective and safe saponin must not only be purified from toxic nonadjuvant components but also from other toxic saponin adjuvants. Because QA-18 is the most predominant saponin in all crude Quillaja bark extracts and Quil-A samples which we have tested, this component is apparently a major contributor to the toxicity associated with either crude extracts and the commercial product although it is clearly not the only purified saponin which is associated with toxicity.

Table 1

Death/Totals*

Dose (ug)	Quil-A	QA-18	QA-7
125	1/5	4/5	N.T.**
250	2/5	5/5	N.T.
500	4/5	N.T.	0/5

*Dose was injected intradermally in CD-1 mice (age = 8 weeks) in a total volume of 200 ul of sterile water. Mice were monitored for 72 hours after injection. Most deaths occurred within 24-36 hours after injection.

**Not tested.

8. The Examiner rejects certain claims in the application as being anticipated by Dalsgaard (1974). The Examiner states that Dalsgaard discloses a purified saponin adjuvant which is less toxic

AIDMAN, STERNE,
:SLER & GOLDSTEIN
ATTORNEYS AT LAW
25 CONNECTICUT AVENUE
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than crude Quillaja extract. We disagree with the conclusion reached by the Examiner. We presented data within the patent application that the product Quil-A described by Dalsgaard is not significantly different from a crude Quillaja extract. Although Dalsgaard calls his product purified, our data shown in the attached declaration Figure 1 and in the patent application clearly indicate otherwise.

9. The Examiner also states that Dalsgaard teaches the use of a saponin adjuvant in a pharmaceutical composition together with an antigen, to induce the production of antibodies to said antigen, thereby enhancing the immune response. Our invention is for substantially pure saponin adjuvants, not a crude saponin mixture as prepared by Dalsgaard. This purity is critical. We now have results which indicate that the purified saponin adjuvants enhance the immune response in a manner which is different from the Quil-A product of Dalsgaard, i.e., the isotype of the antibodies induced. Among the desirable properties of an adjuvant are that the use of it with a vaccine result in a boost not just of total antibody but of a class of antibody which is protective against infectious disease. In Allison's review of adjuvants, supra,, he states:

> As expected, rat and monoclonal antibodies of the IgG2A isotype often confer better protection against infectious agents and tumors than comparable antibodies of other isotypes.

Allison made a systematic comparison of isotypes elicited by

AIDMAN, STERNE,
SLER & GOLDSTEIN
ATTORNEYS AT LAW
:5 CONNECTICUT AVENUE
SHINGTON, D.C. 20036
(202) 466-0800

different adjuvant formulations including Quil-A saponin, alum, Freund's complete and incomplete adjuvants, and SAF-1 and found:

> In mice aluminum salts and saponin produce predominantly antibodies of the IgG1 isotype.

Hence, although it is well documented that Quil-A produces an increase in total antibody titer, it apparently does not induce the isotype maturation which is critical to a protective immune response. By contrast, we have found that saponins QA-7, QA-17, QA-18, and QA-21, purified as described in the patent application, produce predominantly antibodies of the IgG2A isotype. We are enclosing this data as declaration Figure 2 and also a reproduction of a figure from Allison in which the isotype profile of the SAF-1 adjuvant formulation which he considered to be the most efficacious of those which he tested (declaration Figure 3). The data show that these saponins produce an isotype profile which is comparable or better than Allison's best adjuvant. Alum (aluminum salt) was used as a control in our studies and produced predominantly IgG1, a result which was consistent with the result of Allison. Hence, unpurified or partially purified Quillaja saponins such as Quil-A do not elicit the most protective isotype whereas the purified saponins described in our patent application do elicit a desirable isotype in mice, a result which was not anticipated by either Dalsgaard or by Bomford. This refutes the simplistic assumption that to make a partially purified saponin equivalent to a purified saponin in immunization studies only requires that the dose of the partially purified saponin be adjusted

AIDMAN, STERNE,
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ATTORNEYS AT LAW
25 CONNECTICUT AVENUE
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(202) 466-0800

so that the dose of the specific adjuvant-active component(s) are equivalent; clearly other saponins or contaminants which are still present in the partially purified mixture of Dalsgaard are influencing the immune response in a fashion which prevents the development of the most desirable isotype profile. Hence, the type of immune response enhancement induced by the administration of the substantially pure saponin described in the patent application is different than the type of immune response enhancement observed by Dalsgaard.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Charlotte Kensil

CHARLOTTE KENSIL, Ph.D

Dated: 11/21/89

AIDMAN, STERNE,
SLER & GOLDSTEIN
ATTORNEYS AT LAW
25 CONNECTICUT AVENUE
SHINGTON, D. C. 20036
(202) 466-0800

ss # 325-50-7886

Name	Position Title	Birthdate
Charlotte Read Kensil	Section Head Natural Products Chemistry	5-15-54

Institution and Location

University of Illinois Champaign-Urbana	B.S.	1976	Biochemistry
University of California, San Diego	M.S.	1978	Chemistry
University of California San Diego	Ph.D.	1981	Chemistry

Research and Professional Experience

1975-76 University of Illinois, Champaign-Urbana
Undergraduate thesis research; Dr. Michael Glaser, Biochemistry Department. Complementation studies of mutation in E. coli affecting the enzymes adenylate kinase and glycerol 3-phosphate acyltransferase.

1976-81 University of California, San Diego
Thesis research; Dr. Edward A. Dennis, Chemistry Department. Kinetics of phospholipase A_2-catalyzed hydrolysis and of hydroxide-catalyzed hydrolysis of phospholipids dispersed in model membranes.

1981-85 University of Connecticut Health Center
Postdoctoral research; Dr. Philipp Strittmatter, Biochemistry Department. Structural characterization of the microsomal electron transport protein NADH-cytochrome b_5 reductase.

1986-present Cambridge BioScience Corporation, Worcester, MA
Section Head, Natural Products Chemistry
Project leader of Quillaja saponin purification and adjuvant characterization. Project leader of feline leukemia virus vaccine development.

Recent Publications

Marciani, D.J., Hung, C., Cheng, K., and Kensil, C. (1987) Solubilization of inclusion body proteins by reversible n -acylation in Protein Purification: Micro to Macro, p 443-458 (Alan R. Liss, Inc.)

Marciani, D.J., C.R. Kensil, G.A. Beltz, C-H Hung, J. Cronier and A. Aubert. Genetically engineered vaccine against Feline Leukemia Virus: Protective immune response in cats. Submitted for publication.

Relevant Publications

Kensil, C.R., and Dennis, E.A. (1979) Action of cobra venom phospholipase A_2 on the gel and liquid crystalline states of dimyristoyl and dipalmitoyl phosphatidylcholine vesicles. J. Biol. Chem. 254, 5843-5848.

Dennis, E.A., Darke, P.L., Deems, R.A., Kensil, C.R., and Pluckthun, A. (1981) Cobra venom phospholipase A_2: A review of its action toward lipid/water interfaces. Mol. and Cell. Biochem. 36, 37-45.

Kensil, C.R., and Dennis, E.A. (1981) Alkaline hydrolysis of phospholipids and the dependence of their state of aggregation. Biochemistry 21, 6079-6085.

Kensil, C.R., Hediger, M.A., Ozols, J., and Strittmatter, P. (1983) Isolation and partial characterization of the NH_2 -terminal membrane-binding domain of NADH-cytochrome b_5 reductase. J. Biol. Chem. 21, 14656-14663.

Kensil, C.R., and Dennis, E.A. (1985) Action of cobra venom phospholipase A_2 on large unilamellar vesicles: Comparison with small unilamellar vesicles and multibilayers. Lipids 20, 80-83.

Kensil, C.R., and Strittmatter, P. (1986) Binding and fluorescence properties of the membrane domain of NADH -cytochrome-b_5 reductase. J. Biol. Chem. 262, 7316-7321.

Hackett, C.S., Novoa, W.B., Kensil, C.R., and Strittmatter, P. (1988) NADH binding to cytochrome b_5 reductase blocks the acetylation of lysine 110. J. Biol. Chem. 263, 7539-7543.

Not for HIV-2 grant:

Publications

. Roberts, M.F., Otnaess, A.B., Kensil, C.R., and Dennis, E.A. (1978) The specificity of phospholipase A_2 and phospholipase C in a mixed micellar system. J. Biol. Chem. 253, 1252-1257.